REMARKS

Applicant wishes to thank the Examiner for taking the time to discuss the subject case the afternoon of September 12, 2005. In summary, the interview included a discussion of written description issues and proposals for new claims. Based on this discussion applicant believes that claims 108, 110 to 133 and 135 to 151 included herein are allowable.

Claims 108, 110 to 133 and 135 to 151 remain in this case. Claims 109 and 134 have been canceled with prejudice to subsequent revival. Claims 108, 110 to 112, 117, 126, 129, 133, 142 and 143 have been amended. Claims were canceled and certain claims were amended each for the purpose of advancing the case toward allowance and differences between the new claims and the previously pending claims should not be viewed as acquiescence to any of the Examiner's rejections. Applicant believes that this amendment includes no new matter.

Support for certain amended claims is found, for example, at page 7, line 5, of the specification. Page 7, line 5, of the specification refers to Fig. 2 which indicates where PCR primers hybridize to the 2381 bp sequence of SEQ ID NO: 1 for PCR amplification of the approximately 0.5 kb 5' flanking fragment (i.e., nucleotides 1115 to 1626 of Fig. 1 and of corresponding SEQ ID NO: 1).

The Examiner rejects the claims under 35 USC 112, first paragraph, as failing to comply with the written description requirement. Applicant traverses the rejection.

Applicant has amended the claims there by obviating certain aspects of the Examiner's rejection. For example, certain claims have been amended to recite the nucleotide sequence of the 0.5 kb promoter fragment. As discussed in applicant's paper filed April 25, 2005, the 0.5 kb fragment shows high promoter activity (see, page 39, line 32 to page 40, line 1 of the specification). In addition, applicant submits that a practitioner of ordinary skill in the art would reasonably expect that the 336 nucleotide fragment of SEQ ID NO: 2, which is present within the 511 nucleotide sequence of the 0.5 kb fragment, would possess gene expression controlling activity.

The upstream 300 nucleotides have been shown to be important in the gene expression in iFABP genes in other species. For example, Rottman and Gordon 1993 J. Biol. Chem. 268: 11994-12002 (copy included with this response) demonstrate the importance of the rat iFABP gene elements located between nucleotides -277 and -104 in the rat iFABP gene (see, for example, the abstract of Rottman and Gordon). And, as discussed at page 3, line 32 to page 4, line 2 of the

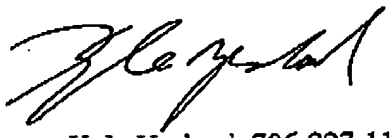
specification, activity of the rat iFABP promoter in the frog suggests conservation of the regulatory mechanism of iFABP expression among vertebrates. See, for example, the Introduction of Beck and Slack 1999 Mechanisms of Development 88: 221-227 (copy included with response). Therefore, it is apparent that the nucleotide sequence of -1 to -336 of the chicken iFABP gene likely contains gene expression controlling activity.

The Examiner rejects claims 142 to 145 under 35 USC 112, second paragraph, as being indefinite for failing to distinctly claim the subject matter which applicant regards as the invention. Applicant traverses the rejection. However, applicant has amended claims 142 and 143 thereby obviating the Examiner's rejection.

Applicant has shown that the requirements of 35 USC 112, first and second paragraphs have been fulfilled and requests that the Examiner pass the above identified application to allowance.

If any issues remain to be addressed in this matter, which might be resolved by discussion, the Examiner is respectfully requested to call applicants' undersigned counsel at the number indicated below.

Respectfully submitted,



Kyle Yesland, 706-227-1170, ext 233
Attorney for Applicants
Reg. No. 45,526
AviGenics, Inc.
Legal Department
111 Riverbend Rd.
Athens, Georgia 30605

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Gut specific expression using mammalian promoters in transgenic *Xenopus laevis*

C.W. Beck*, J.M.W. Slack

Developmental Biology Programme, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

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Abstract

The recent development of transgenic methods for the frog *Xenopus laevis* provides the opportunity to study later developmental events, such as organogenesis, at the molecular level. Our studies have focused on the development of the tadpole gut, where tissue specific promoters have yet to be identified. We have used mammalian promoters, for the genes *elastase*, *pancreatic duodenal homeobox-1*, *transthyretin*, and *intestinal fatty acid binding protein* to drive green fluorescent protein expression in live tadpoles. All of these were shown to drive appropriate tissue specific expression, suggesting that the molecular mechanisms organising the gut are similar in amphibians and mammals. Furthermore, expression from the elastase promoter is initiated in the pancreatic buds before morphological definition becomes possible, making it a powerful tool for the study of pancreatic determination.

Keywords: Gut; Liver; Pancreas; Intestine; *Xenopus laevis*; Transgenic; Promoter; Enhancer; Pancreatic duodenal homeobox-1; XlHbox8; Transthyretin; Elastase; Intestinal fatty acid binding protein

1. Introduction

The frog *Xenopus laevis* has been primarily used to study the mechanisms of early development, and many experiments have involved the injection of synthetic mRNA into early embryos. However, since the injected mRNA eventually decays, the events of later development cannot be easily studied in this way. With the recent development of a method for integrating transgenes into the genome of *X. laevis* and *X. tropicalis* (Kroll and Amaya, 1996; Amaya et al., 1998), *Xenopus* has become an attractive system for studying later development and organogenesis.

Our studies have focused on the development of the tadpole gut, which closely resembles the mammalian gut in its organisation (Chalmers and Slack, 1998). Previously, several regional markers have been described in the *Xenopus* tadpole gut (Wright et al., 1988; Shi and Hayes, 1994; Chalmers and Slack, 1998). However, to date, only a small number of *Xenopus* promoters have been characterised in live embryos using the green fluorescent protein (GFP) reporter system (Kroll and Amaya, 1996; Huang et al., 1999), and none of these drive expression in the gut. In contrast, several well-characterised promoters are available as a result of studies on mammals. Here we show that appropriate regional and tissue specific expression can be achieved in the developing *Xenopus* gut by using several gut-specific mammalian promoters. This shows that the regulation of developmental genes, in addition to the coding regions themselves, has been well conserved during vertebrate evolution.

This work shows that it will be possible to use transgenic *Xenopus* as a quick and simple method to analyse mammalian promoter function. In addition, our results demonstrate that the numerous existing mammalian promoters can be used to drive transgenes in specific patterns in *Xenopus* embryos, thus providing new experimental opportunities for the transgenic technique.

2. Results

2.1. The rat elastase enhancer drives pancreas specific expression in Xenopus

Pancreatic elastase is a serine protease related to trypsin. It is produced by all exocrine cells of the pancreas and is secreted into the duodenal lumen via the common bile duct. The 203 bp regulatory region of the rat pancreatic *elastase I*

* Corresponding author. Tel.: +44-1225-826-826 ext. 5548; fax: +44-1225-826-779.

E-mail address: c.beck@bath.ac.uk (C.W. Beck)

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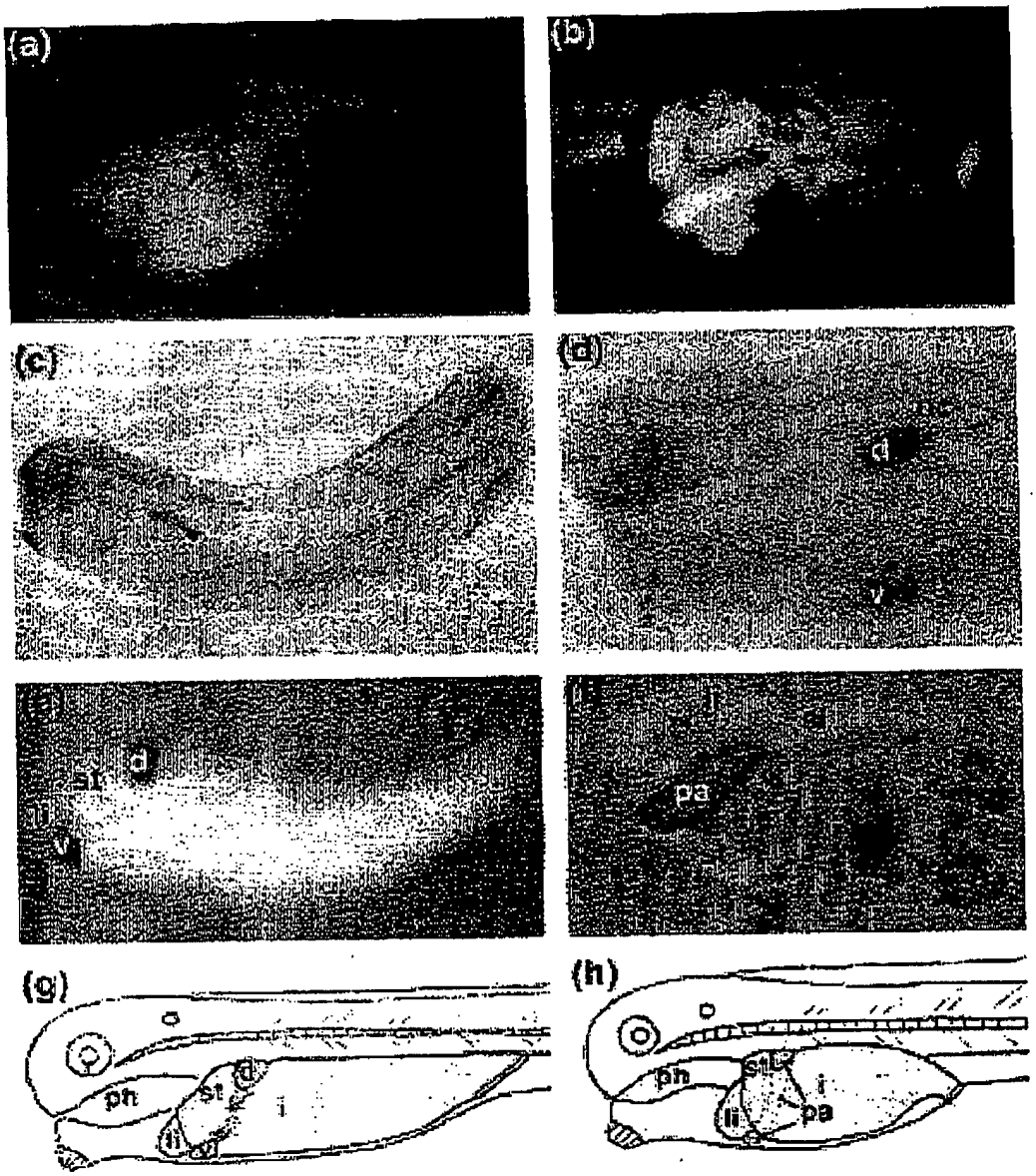

Fig. 1. *Xenopus* tadpoles transgenic for elastase-GFP. (a,b) GFP reporter activity in live tadpoles. Green fluorescence shows where the reporter is active, and yellow colour is due to yolk autofluorescence. (a) Four-day-old tadpole from right hand side, showing GFP limited to the pancreas (arrows). Anterior is to the right. (b) Ventral view of 7-day tadpole with GFP in the pancreas (arrow), anterior is to the right. (c,d) In situ hybridisations to show distribution of GFP mRNA (dark blue) in transgenic embryos, anterior is to the left. (c) Stage 31 embryo, cleared, to show onset of expression in the future dorsal pancreatic bud (d). (d) Stage 33 embryo, cleared, to show onset of expression in the future ventral pancreatic bud (v). Note the close contact between the dorsal bud (d) and the overlying notochord (nc). (e,f), In situ hybridisation to show distribution of GFP mRNA (dark blue) in dissected gut whole mounts. Anterior is to the left. (e) Stage 37 gut, viewed from the left side, to show expression is restricted to the dorsal (d) and ventral (v) pancreatic buds. (f) Expression in the pancreas after fusion of the buds, at 7 days of development, to show persistence of elastase promoter driven expression in all cells of the pancreas (pa). (g,h) Schematic diagrams showing the development and fusion of the pancreatic buds in *Xenopus* embryos based on elastase promoter studies. (g) At stage 37 the dorsal and ventral buds begin to move from the midline, the dorsal pancreas moves to the left and the ventral pancreas to the right. Both buds also begin to extend towards each other, pushing inwards between the stomach and duodenal endoderm. (h) At 3 days of development (stage 41) the pancreatic buds have fused. Subsequent gut coiling then moves the pancreas first to the left side of the embryo, and then to the right side, where it remains from 4 days onwards (not shown). Abbreviations: pa, pancreas; d, dorsal pancreatic bud; v, ventral pancreatic bud; li, liver; st, stomach; si, small intestine; nc, notochord; i, intestine; ph, pharynx.

gene contains an enhancer which drives reporter expression in all exocrine cells of the pancreas (Hammer et al., 1987). Fig. 1 shows reporter expression in *Xenopus* embryos and tadpoles carrying the *elastase-GFP* transgene. GFP fluores-

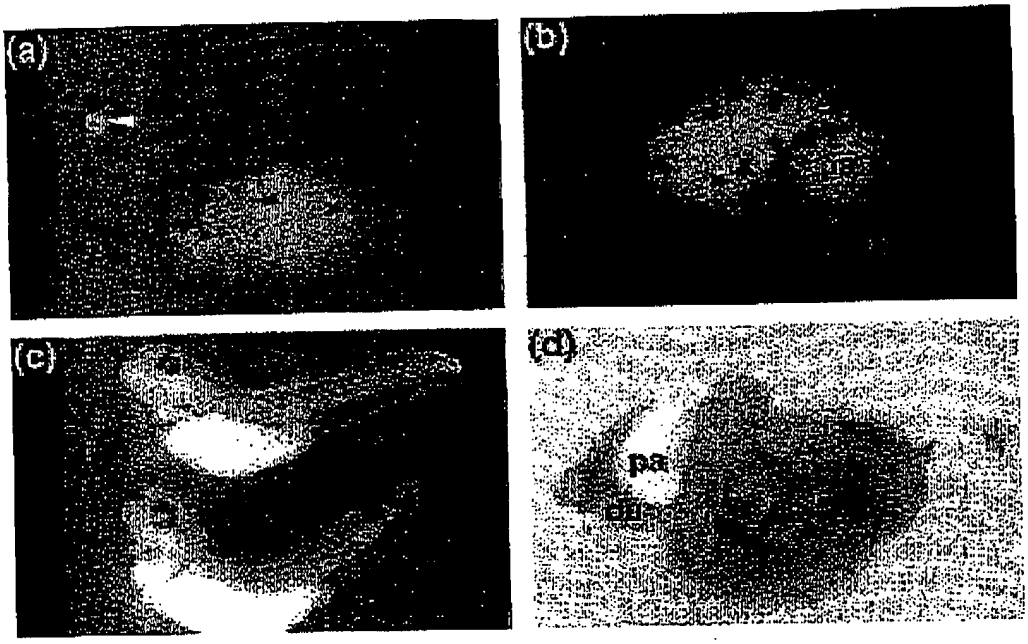

Fig. 2. *Xenopus* tadpoles transgenic for PDX-1-GFP. (a,b) GFP reporter activity in live tadpoles. Green fluorescence shows where the reporter is active, and yellow colour is due to yolk autofluorescence. (a) Three-day-old tadpole from left hand side showing GFP in the pancreatic and duodenal endoderm (black arrow) and lens of the eye (white arrow). Some of the melanocytes, which partially obscure the gut at this stage, have been removed. Anterior is to the left. (b) Four-day-old tadpole viewed from the right hand side to show GFP in the pancreas (pa) but not in the surrounding gut coils. Anterior is to the right. (c) In situ hybridisation to show GFP mRNA in pancreatic buds and duodenum of 3-day-old transgenics (dark blue). View from the left, melanocytes covering the gut have been removed to improve visibility. Anterior is to the left. (d) In situ hybridisation to detect GFP mRNA in whole guts taken from transgenic tadpoles. By 6 days, GFP expression is no longer visible in the pancreas (pa), but low levels of expression are maintained in the duodenum (du). Anterior is to the left. Abbreviations: pa, pancreas; li, liver; du, duodenum.

cence is first seen throughout the pancreatic buds as early as 3 days and continues to be expressed up to 7 days in the pancreas (Fig. 1a,b). We first detect *GFP* mRNA in the dorsal pancreatic bud of transgenic embryos at stage 30–31, around 13 h before the dorsal bud can be defined morphologically at stage 35/36 (Fig. 1c), (Nieuwkoop and Faber, 1967). Expression is restricted to a small group of endodermal cells in the dorsal midline of the anterior midgut, in direct contact with the notochord. It has recently been shown that the notochord is required for development of the dorsal pancreatic bud in the chick embryo (Kim et al., 1997). Expression is also seen prior to morphological definition of the ventral pancreatic bud at stage 37/38. *GFP* mRNA is first detected at stage 33, 10 h before the bud can be defined, in a group of ventral midline cells just posterior to the liver (Fig. 1d).

GFP mRNA was detected throughout the pancreas of later tadpoles transgenic for the *elastase-GFP* construct (Fig. 1e,f). This is expected since in mammals, elastase is made in the acinar (exocrine) cells, which comprise around 98% of the pancreas. In conclusion, the rat elastase-I enhancer appears to be capable of driving appropriate temporal and spatial expression of the reporter in transgenic frogs. Although the expression pattern of endogenous elastase in the frog is currently unknown, the activation of the enhancer prior to morphological definition of the pancreatic endoderm makes it a powerful tool for the study of the earliest steps of pancreas development. Here, we show how the movement of the two pancreatic buds from their original positions in the dorsal and ventral midline as the anterior gut begins to coil, combined with an expansion towards each other leads to formation of the tadpole pancreas (Fig. 1g,h). These drawings are based on observations of live tadpoles transgenic for the elastase enhancer driving GFP.

2.2. *Mouse PDX-1 promoter drives expression in the pancreas and duodenum*

Pancreatic-duodenal homeobox-1 (PDX-1) was originally cloned from mouse as *IPF-1* (Ohlsson et al., 1993) and rat as *STF-1/IDX-1* (Leonard et al., 1993; Miller et al., 1994) and is the mammalian homologue of *XlHbox8* (Wright et al., 1988). Its expression is restricted to the pancreatic and duodenal epithelium of both mammals and frogs (Wright et al., 1988; Ohlsson et al., 1993; Miller et al., 1994; Guz et al., 1995). PDX-1 is essential for initial pancreatic morphogenesis in mice and is also thought to have a later role in transactivation of the insulin gene in beta cells (Jonsson et al., 1994, 1995; Ahlgren et al., 1996; Offield et al., 1996).

In this study we have used a 4.6 Kb *PDX-1* promoter from the mouse gene to drive expression of GFP in transgenic

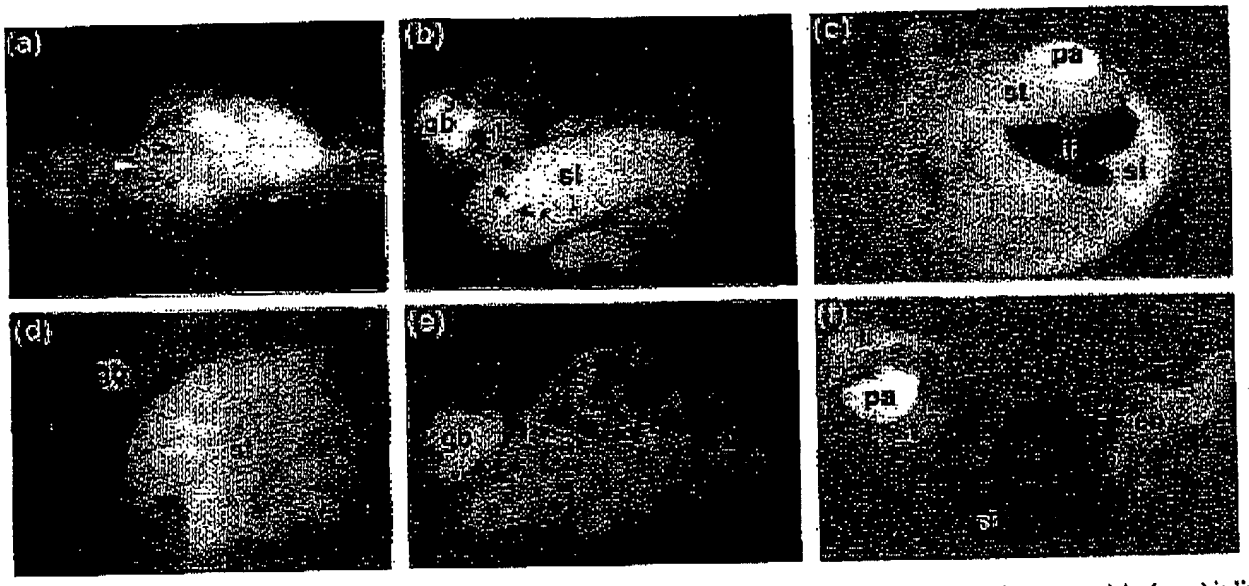

Fig. 9. *Xenopus* tadpoles transgenic for transthyretin-nGFP or IFABP-GFP. (a–c) Transthyretin nGFP transgenics. (a,b) GFP reporter activity (green) in live tadpoles, yolk autofluorescence is yellow, anterior is to the right. (a) Four-day tadpole, ventral view, GFP is present in the liver (arrow) but absent from the rest of the gut, which autofluoresces yellow. (b) Six-day tadpole, ventral/left view to show GFP in the liver (li). The gall bladder (gb), to the left of the liver, fluoresces bright yellow at this stage, as does the small intestine (si). (c) In situ hybridisation to GFP mRNA in whole mount guts from transgenic tadpoles. (c) GFP is expressed exclusively in the liver (dark blue) at 5 days, anterior view. (d–f) *Xenopus* tadpoles transgenic for IFABP-GFP. (d,e) GFP reporter activity (green) in the coils of the small intestine of live 5-day (d) and 7-day (e) tadpoles, both ventral views, anterior to the left. Gall bladder (gb) autofluoresces yellow. (f) In situ hybridisation to GFP mRNA in 7 day whole mount gut. Expression of the reporter is seen in the small intestine (si, dark blue) but absent from the stomach (st), pancreas (pa), liver (li), gall bladder (gb) and colon (co). Abbreviations: gb, gall bladder; li, liver; si, small intestine; pa, pancreas; st, stomach; co, colon.

Xenopus embryos. GFP fluorescence is first detected in the dorsal and ventral pancreatic buds and adjacent duodenal epithelium at 44 h of development (stage 34), before the gut begins to coil (Fig. 2a). Reporter activity in the developing pancreatic buds and pancreas is strikingly similar to the expression of *PDX-1* in the mouse gut (Fig. 2; Ohlsson et al., 1993; Guz et al., 1995) and of native XlHbox8 protein in the frog (Wright et al., 1988). GFP fluorescence is seen throughout the dorsal and ventral pancreatic buds and persists for several days. *PDX-1* expression in the mouse pancreas has been shown to be down regulated as the pancreas differentiates, with expression eventually being restricted to the insulin producing beta cells of the islets of Langerhans (Ohlsson et al., 1993; Miller et al., 1994; Guz et al., 1995). It has therefore been suggested that PDX-1 has a dual role in pancreatic development and function: firstly to enable morphogenesis of the pancreatic epithelium, and secondly to transactivate the insulin gene in the beta cells. The *Xenopus* homologue *XlHbox8* may also have a dual function, as it is similarly down regulated in the majority of the cells of the *Xenopus* pancreas, with expression remaining in only a small percentage of cells (Wright et al., 1988). We examined the distribution of the GFP mRNA in later transgenic tadpole guts by in situ hybridisation, and confirmed that the levels of mRNA are down regulated after 4–5 days of development, with expression remaining only in a small proportion of the cells (Fig. 2d). Note that the GFP fluorescence is still present at this stage, due to perdurance of the protein (not shown). Duodenal expression of *PDX-1* persists in the mouse (Guz et al., 1995) and rat (Miller et al., 1994) and, similarly, we find a low level of expression is maintained as late as 7 days in the duodenum of transgenic tadpoles (Fig. 2d). The distribution of GFP mRNA in transgenics is therefore very similar to that observed for the *Xenopus* homologue of *PDX-1*, *Xlhbox8* (Chalmers and Slack, 1998). In addition to the gut expression, GFP fluorescence is also seen in the lens of the eye from stage 36 onwards. This does not correspond to an endogenous domain of *XlHbox8*, but can be useful because the transgenic and non-transgenic individuals can be immediately distinguished using a fluorescent dissecting microscope.

2.3. The mouse transthyretin regulatory region drives GFP expression in the liver

Transthyretin is an extracellular thyroid hormone-binding protein produced by the liver and choroid plexus of reptiles, birds and mammals. Recently, transthyretin homologues have also been isolated from fish and amphibians, where they are only synthesised in the liver (Yamauchi et al., 1998; Santos and Power, 1999). In mice, hepatocyte specific transcription of transthyretin is known to be regulated by a proximal promoter region and a distal 100 bp

enhancer region located 2 kb upstream of the transcription initiation site. This regulatory region (enhancer and promoter) is sufficient to direct expression to the hepatocytes of transgenic mice (Yan et al., 1990). We have made transgenic *Xenopus* tadpoles using this regulatory region of the mouse transthyretin gene to drive expression of a GFP containing a nuclear localisation sequence (nGFP). Fig. 3 shows that the transgene drives appropriate expression of GFP in the liver of transgenic tadpoles. GFP fluorescence is seen in the posterior foregut from 3–7 days of development, and is located ventrally in the endoderm just posterior to the heart (Fig. 3a,b). Some GFP activity is also seen in the pronephros of transgenic tadpoles. mRNA expression remains throughout the liver in the gut of later tadpoles (Fig. 3c). No expression was seen in the choroid plexus of transgenic tadpoles, in accordance with the endogenous expression pattern (Yamauchi et al., 1998).

2.4. The IFABP promoter from rat drives intestinal GFP expression in Xenopus

Intestinal fatty acid binding protein (IFABP) is expressed in the small intestine of mouse (Green et al., 1992), rat (Alpers et al., 1984), human, (Sweetser et al., 1987) and *Xenopus* (Shi and Hayes, 1994; Chalmers and Slack, 1998). Endogenous expression of *Xenopus IFABP* begins at stage 34, prior to gut coiling, and increases rapidly up to 5 days of development (Shi and Hayes, 1994). Expression has been shown to be restricted to the forming small intestine of guts from 3 day old tadpoles and is excluded from the stomach, accessory organs and colon (Chalmers and Slack, 1998). The rat IFABP promoter has been shown to drive expression of reporters in the small intestine of transgenic mice (Sweetser et al., 1988). We have used this promoter to drive GFP expression in transgenic *Xenopus*. In live embryos, GFP fluorescence is first seen at 4–5 days of development, when it is localised to the intestinal epithelium of the coiled gut (Fig. 3d). GFP activity increases in intensity and by 7 days is seen throughout the outer gut coil, but is absent from the stomach, liver and pancreas (Fig. 3e). In situ hybridisation for the *GFP* mRNA in whole gut preparations at 6–7 days confirmed the absence of reporter expression in the colon, which forms the posterior half of the inner gut coil, and is therefore not visible in live embryos (Fig. 3f). The spatial expression of the reporter gene therefore corresponds with that of the endogenous gene (Shi and Hayes, 1994; Chalmers and Slack, 1998).

3. Discussion

We have examined expression at protein and mRNA levels from four gut specific mammalian promoters in transgenic *Xenopus* embryos and tadpoles. In each case, the temporal and spatial expression of the reporter has been shown to match that shown previously in transgenic mice. Where the expression patterns of the endogenous *Xenopus* homologues are known, they also correspond well to the reporter expression (Wright et al., 1988; Shi and Hayes, 1994; Chalmers and Slack, 1998; Yamauchi et al., 1998).

This work represents, to our knowledge, the first use of mammalian promoters to make *Xenopus* transgenics, and the results have significance in three respects. Firstly, they show at the level of gene regulation that the mechanisms of gut development are well conserved between mammals and *Xenopus*. This is of some evolutionary interest and also means that *Xenopus* can be considered a valid model for studies of gut development as the results are likely to apply also to higher vertebrates.

Secondly, it means that *Xenopus* transgenics can be used as a rapid and informative test of promoter activity. Compared with transgenic mice, *Xenopus* offer many technical advantages. It is possible to make a large number of transgenic individuals in 1 day. If GFP reporters are used, the transgenics can be distinguished from non-transgenics by simple dissecting microscope observation. It is possible to observe the reporter activity in live embryos at stages which are inaccessible in mammals, and the transparent nature of the tadpole allows visualisation of reporter activity in deep tissues, such as the gut.

Finally, the fact that mammalian promoters can be used is of considerable importance for designing experiments on the development of *Xenopus* itself. As numerous mammalian promoters are already available, it will avoid the need for the cloning of endogenous promoters for misexpression studies. The instant availability of a large number of promoters means that the *Xenopus* transgenic system can rapidly be brought to bear on the study of later developmental events, such as gut development.

4. Materials and methods

4.1. Transgenic Xenopus laevis

Transgenic *Xenopus laevis* embryos were made using the restriction enzyme mediated integration (REMI) method of Kroll and Amaya (1996) with modifications as suggested by Martin Offield (pers comm). Briefly, 5 μl of high speed egg cytosolic extract was used in each REMI reaction rather than 25 μl, and $MgCl_2$ reduced accordingly to maintain 5 mM final concentration. Eggs were dejellied in 1×NAM salts, 1.6 mM DTT, and 200 mM Tris (pH 8.8), and sperm dilution buffer was made with 250 μM spermidine and 125 μM spermine (Sigma). Embryos were cultured as in (Pownall et al., 1998), using NAM to replace MMR throughout. Fifty micrograms of transgene DNA was digested to completion with the appropriate enzyme, extracted with phenol/chloroform, gel purified by electroelution and cleaned using Wizard PCR prep DNA purification columns (Promega). DNA in the range of 50–500 ng/μl was successfully used to make transgenics. We typically obtain 2–6% viable transgenic tadpoles using this protocol,

which corresponds to 16–50 transgenics from each REMI reaction.

Embryos up to stage 41 (3 days of development) were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967), and subsequently staged by the number of days of development, as determined for gut development in Chalmers and Slack (Chalmers and Slack, 1998). Four days corresponds to embryonic stage 44/45; 5 days to stage 45/46; 6 days to stage 46, and 7 days to stage 46/47. All transgenic embryos were terminated at 7 days, before feeding began.

4.2. Transgene constructs

PDX-1-GFP in PIT2 vector was a kind gift of Chris Miller, Genetics Institute. The construct contained 4.6 kb of the mouse PDX-1 promoter, and the transgene (6.5 kb) was excised using flanking Not I restriction sites. Elastase promoter was a kind gift of Galvin Swift, TX, USA. The 203 bp rat elastase I promoter (gb: L00112) was subcloned into PIT2 and the 2.1 kb transgene was excised using flanking *NotI* sites. Transthyretin-nucGFP was a kind gift of David Tosh, Bath. The construct contains the 3 kb upstream region containing both the enhancer and promoter regions of mouse transthyretin (gb: M19524) in pcDNA3 (Invitrogen) containing nuclear GFP and was linearised with Apa I to make the transgene. IFABP promoter was a kind gift of Indira Mysorekar, Washington University. The 1.2 kb rat IFABP promoter (gb: M18080) was subcloned into PIT2 and the 3 kb transgene was excised using *NotI*.

4.3. Visualisation of GFP in live tadpoles

Transgenic tadpoles were anaesthetised in 1/2000 MS-222 (3-aminobenzoic acid ethyl ester, Sigma) and visualised using a Leica Fluo III fluorescent dissecting microscope with a GFP2 filter set.

4.4. Whole mount in situ hybridisation

GFP mRNA distribution was assayed using in situ hybridisation with an antisense GFP digoxygenin labelled RNA probe as Harland (Harland, 1991), modifications as Pownall et al. (1996). Whole gut dissections were performed as in Chalmers and Slack (1998).

Acknowledgements

We thank Betsy Pownall, Enrique Amaya and Martin Offield for advice on transgenic *Xenopus* methods, Andrew Chalmers for advice on tadpole gut development and Chris Miller and David Tosh for providing mammalian promoters. This work was supported by grants from Genetics Institute and the Wellcome Trust.

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Comparison of the Patterns of Expression of Rat Intestinal Fatty Acid Binding Protein/Human Growth Hormone Fusion Genes in Cultured Intestinal Epithelial Cell Lines and in the Gut Epithelium of Transgenic Mice*

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Jeffrey N. Rottman and Jeffrey I. Gordon‡

From the Departments of Molecular Biology and Pharmacology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

The intestinal fatty acid binding protein gene (*Fabpi*) provides a good model system for studying how gene transcription is regulated in enterocytes as a function of their differentiation program and location along the duodenal-to-colonic axis. We have compared and contrasted the transcriptional activity of four fusion genes composed of elements from the 5′-nontranscribed domain of rat *Fabpi* linked to the human growth hormone gene (I-FABP/hGH) in transgenic mice and in five primate epithelial cell lines derived from intestine, liver, kidney, and cervix. Nucleotides −103 to +28 of rat *Fabpi* are able to direct appropriate lineage-specific and geographic patterns of hGH expression in transgenic mice. I-FABP$^{-103\ to\ +28}$/hGH is preferentially expressed in Caco-2 cells, which emulate some of the features of differentiated small intestinal enterocytes after they reach confluence. However, other I-FABP/hGH fusion genes that exhibit differentiation-dependent changes in their expression along the crypt-to-villus axis do not manifest the same pattern of differentiation-dependent change in activity in this cell line. Correlation of their patterns of expression *in vivo* and *ex vivo* suggest that nonproliferating Caco-2 cells mimic some of the features of the transcriptional regulatory environment of enterocytes located in the upper crypt.

Nucleotides −103 to +28 of rat *Fabpi* contain one copy of a repeated 14-base pair element that is conserved in the orthologous mouse and human genes and represented in several other homologous and nonhomologous genes, which are expressed in villus-associated enterocytes. This element binds to two members of the steroid hormone receptor superfamily of transcription factors produced in enterocytes and Caco-2 cells: hepatic nuclear factor-4 (HNF-4) and apolipoprotein regulatory protein-1 (ARP-1). Co-transfection studies performed in Caco-2 cells and in a monkey kidney cell line (CV-1) that lacks endogenous pools of ARP-1 and HNF-4 suggest that ARP-1 and HNF-4 can function to activate I-FABP$^{-103\ to\ +28}$/hGH^{+2} through their interactions with the 14-base pair element. This activation appears to be affected by elements located between nucleotides −277 and −104 and other transcription factors.

There are several reasons why the intestinal fatty acid binding protein gene (*Fabpi*)[1] is a useful model for studying the mechanisms that regulate establishment and maintenance of cell lineage-specific patterns of transcription along the crypt-to-villus and duodenal-to-colonic axes of the gut. *First*, expression of mouse, rat, and human *Fabpi* is confined to the enterocyte (Sweetser *et al.*, 1987; Green *et al.*, 1992; Cohn *et al.*, 1992), one of four principal epithelial cell types derived from the multipotent stem cell located near the base of each intestinal crypt (reviewed in Potten and Loeffler (1990) and Gordon *et al.* (1992)). *Fabpi* is activated in differentiating members of the enterocytic lineage after they complete their last passage through the cell cycle and are translocated from the upper crypt to the bases of adjacent small intestinal villi (Shields *et al.*, 1986; Green *et al.*, 1992; Cohn *et al.*, 1992). Expression is sustained as enterocytes complete their differentiation program during a rapid, well organized, upward migration to the apical extrusion zone of each villus. *Fabpi* is also expressed in enterocytes as they exit colonic crypts and migrate to the colonic homolog of small intestinal villi, the hexagonal shaped surface epithelial cuff that surrounds the orifice of each colonic "gland" (Schmidt *et al.*, 1985). *Second*, the steady state level of *Fabpi*'s mRNA and protein products varies as a function of the location enterocytes occupy along the cephalocaudal axis of the gut. Highest cellular concentrations occur in the jejunum with levels falling progressively to the proximal duodenum and midcolon (Sweetser *et al.*, 1988; Cohn *et al.*, 1992). These axial differences in *Fabpi* expression are established at the time of the gene's induction during cytodifferentiation of the gut endoderm to an epithelial monolayer in fetal life (Rubin *et al.*, 1989, 1991; Cohn *et al.*, 1992). These regional differences in *Fabpi* expression are sustained throughout life despite continuous and rapid renewal of the intestinal epithelium (Al-Nafussi and Wright, 1982; Wright and Irwin, 1982). *Third*, studies with fetal jejunal, ileal, and colonic isografts indicate that the lineage-specific, differentiation-dependent, and regional patterns of rat and mouse *Fabpi* expression are not influenced by luminal contents (Rubin *et al.*, 1991, 1992). *Fourth*, *Fabpi* is a member of a family of homologous genes that exhibit distinct cephalocaudal pat-

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‡ To whom correspondence should be addressed: Dept. of Molecular Biology and Pharmacology, Washington University School of Medicine, Box 8103, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-7243; Fax: 314-362-7058.

[1] The abbreviations used are: *Fabpi*, intestinal fatty acid binding protein gene; hGH, human growth hormone; MT, metallothionein; HNF-4, hepatic nuclear factor-4; ARP-1, apolipoprotein AI regulatory protein-1; kb, kilobase(s); bp, base pair(s).

terns of expression in the enterocytic lineage (Sacchettini *et al.*, 1990; Cohn *et al.*, 1992). These differences provide an opportunity to study how genes that have evolved from a common ancestor are able to modulate their expression in a particular cell lineage as a function of location along the duodenal-to-colonic axis.

Transgenic mice containing rat *Fabpi*/human growth hormone (hGH) fusion genes have been used to map cis-acting elements in *Fabpi* that control its cellular, geographic, and temporal patterns of expression (Sweetser *et al.*, 1988; Cohn *et al.*, 1992). The results of these studies are summarized in Fig. 1. Remarkably, nucleotides −103 to +28, which are highly conserved in the mouse, rat, and human *Fabpi* genes (Green *et al.*, 1992), contain elements that are sufficient to appropriately initiate transgene expression in late fetal life, confine reporter production to members of the enterocytic lineage, and to generate a duodenal-to-colonic gradient of hGH mRNA and protein accumulation that mimics that of mouse *Fabpi* (Cohn *et al.*, 1992). The shape of this cephalocaudal gradient is influenced by both positive and negative cis-acting sequences, *e.g.* elements positioned between nucleotides −1178 and −278 enhance expression in the ileum and proximal colon while those located between −277 and −185 function as suppressors in these segments of the gut (Cohn *et al.*, 1992) (Fig. 1). Progressive deletions of the 5′-nontranscribed domain of *Fabpi* also disclosed elements that regulate differentiation-dependent transcription of this gene, *e.g.* I-FABP$^{-277\ to\ +28}$/hGH is appropriately activated in enterocytes as they exit intestinal crypts while removal of nucleotides −277 and −185 (yielding I-FABP$^{-184\ to\ +28}$/hGH) results in precocious expression of the hGH reporter in proliferating and nonproliferating epithelial cells located in the upper half of duodenal, jejunal, ileal, and colonic crypts (Cohn *et al.*, 1992).

Although recent reports have described methods for preparing primary cultures of gut epithelial cells (Evans *et al.*, 1992) or conditionally immortalized cell lines (Whitehead *et al.*, 1993), none of these systems have been shown to be capable of recapitulating the complex geographic differences in the enterocytic differentiation program or the terminally differentiated state. We have now surveyed expression of a variety of *Fabpi*/hGH fusion genes in established epithelial cell lines derived from intestinal and extraintestinal tissues. This has provided an opportunity to compare and contrast the activities of a panel of recombinant DNAs in transgenic animals and in cultured cells and determine whether the latter contain a transcriptional regulatory environment that could faithfully support, *ex vivo*, some features of *Fabpi* expression observed *in vivo*. In addition, we have used these cell lines to identify trans-acting factors that affect the transcriptional activity of nucleotides −103 and +28 of rat *Fabpi*.

EXPERIMENTAL PROCEDURES

Construction of Recombinant DNAs—pIFhGH1 (Sweetser *et al.*, 1988) contains nucleotides −1178 to +28 of rat *Fabpi* (Sweetser *et al.*, 1987) linked to the human growth hormone gene starting at its nucleotide +3 (Seeburg, 1982). This recombinant DNA was designated I-FABP$^{-1178\ to\ +28}$/hGH^{+3}. I-FABP$^{-277\ to\ +28}$/hGH^{+3} was obtained by digesting pIFhGH1 with *Eco*RI yielding a 2.4-kb fragment, which was cloned into the *Eco*RI site of pBluescript SKII (Promega, Madison, WI). I-FABP$^{-184\ to\ +28}$/hGH^{+3} was obtained from I-FABP$^{-277\ to\ +28}$/hGH^{+3} by digesting I-FABP$^{-277\ to\ +28}$/hGH^{+3} with *Est*EII, blunting the ends of the resulting reaction products with the Klenow fragment of DNA polymerase I, incubating the material with *Eco*RI, and ligating the 2.3-kb restriction fragment to the *Sma*I and *Eco*RI sites present in the polylinker of pBlueScript SKII. I-FABP$^{-103\ to\ +28}$/hGH^{+3} was produced by cleaving I-FABP$^{-277\ to\ +28}$/hGH^{+3} with *Sph*I and subcloning the 1.9-kb fragment into the *Sph*I site of pUC19 (Sambrook *et al.*, 1989). I-FABP$^{82/-277\ to\ +28}$/hGH^{+3} was produced by placing two copies of the sequence 5′-AGCTTTGAACTTCGAACTTA-3′ into the *Hin*dIII site of the polylinker of I-FABP$^{-277\ to\ +28}$/hGH^{+3} (this site is located 8 bases upstream from nucleotide −277 of rat *Fabpi*). The dideoxynucleotide sequencing method (Sanger *et al.*, 1977) was used to confirm that the desired elements were present in the recombinant plasmid. MT/hGH^{+3} (pXGH5 from Nichols Institute, San Juan Capistrano, CA) contains 1.8 kb of the mouse metallothionein promoter linked to the coding sequence of the hGH gene. C3P-TK-hGH^{+3} was constructed by taking the HNF-4/ARP-1/Ear3/COUP-TF binding site spanning nucleotides −86 to −74 of the human apolipoprotein (apo) CIII gene (Mietus-Snyder *et al.*, 1992), adding *Hin*dIII linkers to each end (yielding the sequence 5′-GCAGGTGACCTTTGCCCA-GCGC-3′), and placing the double-stranded oligodeoxynucleotide into the *Hin*dIII site beginning at base −109 of the *Herpes simplex* virus thymidine kinase gene contained in pTKGH (Nichols Institute). cDNAs encoding hepatic nuclear factor-4 (HNF-4, Sladek *et al.* (1990)) and apolipoprotein regulatory protein-1 (ARP-1, Ladias and Karathanasis (1991)) were subcloned as *Eco*RI fragments into the *Eco*RI site of the eukaryotic expression vector, pMT2 (Sambrook *et al.*, 1989) yielding pMT2-HNF-4 and pMT2-ARP-1, respectively (Ladias *et al.*, 1992).

Cell Culture—HepG2, CV-1, SK-Hep, HeLa, and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained at 37 °C under an atmosphere of 5% CO_2, 95% air in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO/BRL) and penicillin/streptomycin (final concentration: penicillin, 5 units/ml; streptomycin, 5 μg/ml). Caco-2 cells were also obtained from the ATCC and maintained as above except that the concentration of fetal calf serum was 20% rather than 10%. Total cellular RNA was prepared from pre- and/or postconfluent cells using guanidine isothiocyanate (Chirgwin *et al.*, 1979), and the pattern of expression of the intact, endogenous *Fabpi* gene was determined by RNA blot hybridization (Sambrook *et al.*, 1989).

Transient Transfection Experiments—Cells were seeded onto polystyrene plates at a density of 2×10^5 cells/30-mm well. Cells were transfected 36 h after plating with 5 μg of plasmid DNA using the calcium phosphate co-precipitation method (Graham and van der Eb, 1973). To control for variations in transfection efficiency, 1 μg of pRSV-β-Gal DNA (Edlund *et al.*, 1985) was co-transfected together with each promoter/hGH^{+3} construct. Sixteen hours later, cell monolayers were washed twice in phosphate-buffered saline, and 2 ml of fresh medium were added. Aliquots of medium were withdrawn 48 h later and cells removed by centrifugation at 17,000 × *g* for 3 min. Cells were then lysed *in situ* using three cycles of freezing/thawing. Cell lysates were assayed for β-galactosidase activity according to a protocol described in Sambrook *et al.* (1989). hGH levels were measured in the medium using a radioimmunoassay kit (Nichols Institute). Hormone levels were expressed as nanograms of hormone/milliliter of supernatant and then normalized to total cellular β-galactosidase activity to control for variations in transfection efficiency between and within experiments. (Control experiments indicated that the antibodies used in the radioimmunoassay kit do not cross-react with bovine growth hormone contained in fetal calf serum.) Levels of I-FABP/hGH^{+3} expression in each cell line were also normalized relative to the levels of hGH production in parallel cultures of cells co-transfected with MT/hGH^{+3} and pRSV-β-Gal to allow for direct comparison of results obtained with different transfected cell lines.

Experiments Involving Stably Transfected Caco-2 Cells—I-FABP/hGH^{+3} DNA and pRSV-Neo DNA (Kim and Wold, 1985; molar ratio of DNAs, 40:1) were used to transfect Caco-2 cells using the protocol described above. Cells were subsequently incubated with Dulbecco's modified Eagle's medium supplemented with fetal calf serum (20%) and G418 (final concentration, 800 μg/ml, GIBCO/BRL). Once stable, G418-resistant populations were obtained, 10^6 pooled cells were plated in a T25 flask. Five-ml aliquots of culture medium were obtained every 3 days as the time cells were re-fed. These samples of medium were then subjected to centrifugation at 17,000 × *g* to remove residual cells, and hGH concentrations were measured in the resulting supernatants (cleared medium) using the radioimmunoassay kit. The point at which confluence was first achieved was designated day 0. The level of expression of a given I-FABP/hGH^{+3} fusion gene at each time point surveyed was referenced to the level of expression of that gene on day 0.

Electrophoretic Mobility Gel Shift Assays—CV-1 and Caco-2 nuclear extracts were prepared using a modification (Ausubel *et al.*, 1991) of the procedure of Dignam *et al.* (1983) with final dialysis against 100 mM KCl. For production of crude preparations of HNF-

4 and ARP-1, pMT2-HNF-4 and pMT2-ARP-1 were introduced into COS-7 cells by calcium phosphate co-precipitation. Cell lysates were prepared 48 h later by subjecting cells to three cycles of freezing/thawing in a solution of phosphate-buffered saline containing 0.2 mM phenylmethylsulfonyl fluoride. Lysates were subjected to centrifugation at 17,000 × *g* to remove insoluble cellular debris. Protein concentrations were determined in the resulting supernatants using the method of Bradford (1976).

DNA fragments used for electrophoretic mobility gel shift assays were purified from agarose gels with GeneClean (MidWest Scientific, Valley Park, MO) and their 3′ ends labeled using [α^{32}P]dATP and the Klenow fragment of DNA polymerase I (Sambrook *et al.*, 1989). The purified probe (25 fmol) was added to a reaction mixture (final volume, 15 μl) containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 1 μg of poly(dI-dC), nuclear extract (1 μg of total protein), and, where indicated, an unlabeled oligodeoxynucleotide competitor (1.25 pmol). Following a 15-min incubation on ice, the mixture was fractionated by electrophoresis through non-denaturing polyacrylamide gels using the buffer systems described in the legend to Fig. 3. Gels were dried and exposed to Kodak XAR film at −80 °C.

Methylation Interference Assays—These assays were conducted using Caco-2 nuclear extracts or crude preparations of HNF-4 or ARP-1 obtained from transfected COS-7 cells. The −103 to +28 fragment of *Fabpi* was obtained by SphI and BamHI digestion of I-FABP$^{-103\text{ to }+28}$/hGH^{+3}. The BamHI site was labeled on the coding strand using [α^{32}P]dATP and the Klenow fragment of DNA polymerase I. The DNA was partially methylated with dimethyl sulfate (Ausubel *et al.*, 1991) and added to a reaction mixture composed of the reagents described above for the electrophoretic mobility shift assays (except that we increased the scale of the reaction 5–10-fold). After a 15-min incubation on ice, the mixture was subjected to polyacrylamide gel electrophoresis (buffer: 45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). The unfixed gel was exposed to Kodak XAR film at 4 °C to visualize protein-DNA complexes as well as free probe. These labeled bands were subsequently electroeluted onto NA-45 DEAE paper according to a method suggested by the manufacturer (Schleicher and Schnell). Following a 15-min incubation at 90 °C in a solution of 10 mM sodium phosphate, 1 mM EDTA (pH 8.0), base-specific (G>>A) cleavage was accomplished using 0.1 N NaOH. After a 30-min incubation at 90 °C, the reaction products were recovered by ethanol precipitation and fractionated by electrophoresis through a 15% polyacrylamide gel containing 8 M urea. The gel was then fixed in methanol/acetic acid, dried, and subjected to autoradiography at −80 °C.

RESULTS AND DISCUSSION

Epithelial Cell Type-specific Expression of I-FABP/hGH^{+3} Constructs—Five different primate epithelial cell lines were included in our study. Caco-2 cells are derived from a human colon adenocarcinoma. After achieving confluence they are able to complete a differentiation program that results in the acquisition of some features of human small intestinal enterocytes (Pinto *et al.*, 1983; Rousset 1986). CV-1 cells are derived from the kidney of an African Green monkey (Jensen *et al.*, 1964). HepG2 cells are derived from a human hepatoma and express a broad range of gene products that are also produced in normal human hepatocytes (Aden *et al.*, 1979). Human SK-Hep-1 cells are of hepatic origin but have a less differentiated phenotype than HepG2 cells (Fogh and Trempe, 1975). The HeLa cell line was established from a human cervical adenocarcinoma (Gey *et al.*, 1952). Proliferating cultures of these cell lines do not contain detectable levels of I-FABP mRNA as determined by RNA blot hybridization analyses (Sweetser *et al.* (1987) and data not shown).

These five cell lines were transiently transfected prior to achieving confluence with each of the four I-FABP/hGH^{+3} DNAs shown in Fig. 1. Levels of hGH in all cell lines transfected with I-FABP$^{-1178\text{ to }+28}$/hGH^{+3} were ≤1% of that obtained with the reference control vector MT/hGH^{+3} (Table I). Successive deletions of the 5′ nontranscribed domain of I-FABP$^{-1178\text{ to }+28}$/hGH^{+3} resulted in progressive *increases* in expression in proliferating Caco-2 cells. A very pronounced increase in expression was observed in Caco-2 cells when nucleotides −277 to −185 of *Fabpi* were removed (Table I). This finding is consistent with results obtained in transgenic mice, *i.e.* I-FABP$^{-277\text{ to }+28}$/hGH^{+3} is only expressed in villus-associated enterocytes while I-FABP$^{-184\text{ to }+28}$/hGH^{+3} is precociously activated in proliferating and nonproliferating epithelial cells located in the upper half of small intestinal crypts (see Introduction and Cohn *et al.* (1992)).

I-FABP$^{-184\text{ to }+28}$/hGH^{+3} expression is restricted to villus-associated enterocytes (Cohn *et al.*, 1992). In contrast to these *in vivo* results, deletion of nucleotides −277 to −185 is associated with an approximately 10-fold increase in hGH levels in HepG2 and SK cell culture medium (from 1% of the concentration documented in MT/hGH^{+3} transfected controls to 10–11%, *cf.* Table I). Nonetheless, the absolute levels of hGH in these hepatocyte-like cell lines are 4–6-fold lower than those observed in Caco-2 cells. Moreover, only minimal changes in expression occur in CV-1 and HeLa cells when nucleotides −277 to −185 of rat *Fabpi* are removed (Table I).

Further deletions from the 5′ nontranscribed domain of rat *Fabpi* result in even greater degrees of expression in proliferating Caco-2 cells; medium hGH concentrations in I-FABP$^{-103\text{ to }+28}$/hGH^{+3} transfected cells are 5 times higher than in cells containing I-FABP$^{-184\text{ to }+28}$/hGH^{+3} (Table I) and more than 20-fold higher in absolute terms than in any other of the established epithelial cell lines. In contrast, enterocytic levels of hGH mRNA and protein fall in transgenic mice when nucleotides −184 to −104 are deleted even though lineage specificity is retained (Cohn *et al.*, 1992).

Changes in I-FABP/hGH^{+3} Expression during Caco-2 Differentiation Suggest That Postconfluent Caco-2 Cells Resemble Epithelial Populations Located in the Upper Crypt—Within 10 days after achieving confluency, Caco-2 cells undergo a differentiation program that involves formation of intracellular tight junctions (as assessed by changes in electrical resistance across monolayers grown on filters (Grasset *et al.*, 1984) and marked elevations in the levels of several brush border hydrolases and transport proteins (Pinto *et al.*, 1983; Mahraoui *et al.*, 1992). An experiment was performed to examine the relationship between the proliferation and differentiation programs of Caco-2 cells and their ability to support expression of I-FABP/hGH^{+3} fusion genes. Pools of cells containing stably integrated copies of I-FABP$^{-1178\text{ to }+28}$/hGH^{+3}, I-FABP$^{-184\text{ to }+28}$/hGH^{+3}, or MT/hGH^{+3} were plated at subconfluent densities and hGH production monitored every 3 days after cultures reached confluency (day 0). Cells were re-fed with fresh medium after each sampling. Under the conditions of this experiment, any changes in the level of hGH observed in medium harvested from a population of *nonreplicating*, stably transfected cells should reflect changes in reporter production associated with differentiation.

The concentration of hGH in the medium of cells containing I-FABP$^{-184\text{ to }+28}$/hGH^{+3} increased 3–4-fold within 3 days after reaching confluence (Fig. 2). These cells assume many characteristics of the differentiated phenotype between 3 and 12 days after confluence (as defined by the appearance of brush border-specific sucrose-isomaltase and tight junctions). During this interval, the concentration of hGH fell progressively, reaching levels that were 50% of those noted on day 0 (Fig. 2). In contrast, the relative levels of hGH production in Caco-2 cells stably transfected with I-FABP$^{-1178\text{ to }+28}$/hGH^{+3} or MT/hGH^{+3} remained essentially constant during this 12-day period (Fig. 2).

The behavior of I-FABP$^{-184\text{ to }+28}$/hGH^{+3} in Caco-2 cells is consistent with the high levels of expression of I-FABP$^{-184\text{ to }+28}$/hGH^{+3} observed in crypt epithelial cells that

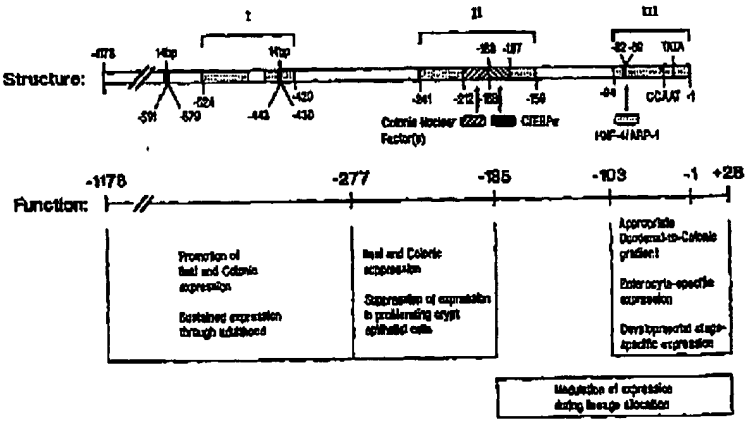

FIG. 1. **Summary of the I-FABP/hGH^{+3} DNAs characterized in transgenic mice and used for transfection of cultured primate epithelial cell lines.** Domains I–III represent three highly conserved elements present in the orthologous rat, mouse, and human *Fabpi* genes (Green *et al.*, 1992). Gel shift mobility assays identified a 24-bp sequence spanning nucleotides −241 to −188 of rat *Fabpi* that binds proteins present in colonic but not proximal small intestinal nuclear extracts (Cohn *et al.*, 1992). DNase I footprinting studies employing *Escherichia coli*-derived C/EBPα (Landschulz *et al.*, 1988) and nucleotides −277 to +28 of rat *Fabpi* disclosed a single protected site between nucleotides −188 and −167 (Cohn *et al.*, 1992). Computer-assisted sequence analyses of the three orthologous *Fabpi* genes revealed several copies of a 14-bp element (Sweetser *et al.*, 1987). This element is also represented in the homologous *Crbp2* gene whose lineage-specific and regional patterns of expression mimic those of *Fabpi*. This figure has been adapted from Cohn *et al.* (1992).

TABLE I

Relative levels of expression of I-FABP/hGH^{+3} DNAs in transiently transfected, proliferating cultured primate epithelial cell lines

Proliferating cell lines were co-transfected with the indicated I-FABP/hGH^{+3} construct together with pRSV/β-galactosidase DNA. hGH levels in media harvested 64 h after transfection were normalized to β-galactosidase activity present in cells lysates. Expression of each I-FABP/hGH^{+3} DNA in each cell type has been presented as a percentage of MT/hGH^{+3} expression in the same cell type. All transient transfection experiments were repeated at least three times and the results averaged. See "Experimental Procedures" for further details.

Construct	Cell line				
	Caco-2	CV-1	HepG2	SK	HeLa
MT/hGH^{+3}	100%[a]	100%[b]	100%[c]	100%[d]	100%[e]
I-FABP$^{-1178\ to\ +28}$/hGH^{+3}	1 ± 1	0	0	0	0
I-FABP$^{-277\ to\ +28}$/hGH^{+3}	10 ± 3	1 ± 0	1 ± 0	1 ± 0	1 ± 0
I-FABP$^{-184\ to\ +28}$/hGH^{+3}	110 ± 26	2 ± 1	11 ± 1	10 ± 1	2 ± 1
I-FABP$^{-103\ to\ +28}$/hGH^{+3}	490 ± 125	0	4 ± 1	5 ± 1	2 ± 2

[a] 100% = 5–51 ng of hGH/ml of supernatant.
[b] 100% = 5–60 ng of hGH/ml of supernatant.
[c] 100% = 4–21 ng of hGH/ml of supernatant.
[d] 100% = 8–14 ng of hGH/ml of supernatant.
[e] 100% = 7–40 ng of hGH/ml of supernatant.

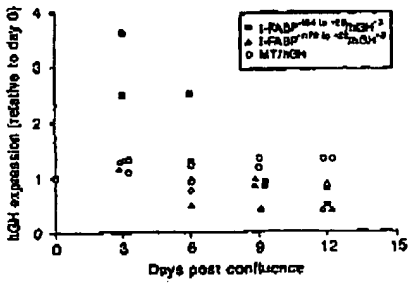

FIG. 2. **Expression of I-FABP/hGH^{+3} DNAs in stably transfected Caco-2 cells as a function of time after achieving confluency.** Caco-2 cells were co-transfected with the indicated DNA and pRSV-Neo. Following a selection for resistance to the aminoglycoside G418, 10^5 cells were plated in a T25 flask. Day 0 represents the point at which confluence was achieved. Medium was subsequently replaced every 2 days. The level of I-FABP/hGH^{+3} expression was determined by measuring the concentration of hGH in medium at the time it is replaced. Hormone levels are referenced to the levels observed on day 0 in the same flask.

have just exited the cell cycle and in members of the enterocytic lineage that are commencing their differentiation during translocation from the upper crypt to the base of adjacent villi (Cohn *et al.*, 1992). There is no information about the transcription rates of *Fabpi* or I-FABP/hGH^{+3} as a function of cellular location along the crypt-to-villus axis. Therefore, we do not know what an "appropriate" change in hGH production would be in a such a cell culture system.[2]

The apparent failure of I-FABP$^{-1178\ to\ +28}$/hGH^{+3} to show a change in hGH production rate during progression through the Caco-2 differentiation program may reflect a number of underlying mechanisms. For example, differentiating, postconfluent Caco-2 cells may not synthesize transcription factors that interact with positive cis-acting regulatory sequences located upstream of nucleotide −277 of rat *Fabpi*. This may also explain the inactivity of the intact, endogenous human *Fabpi* gene in postconfluent Caco-2 cells. Alternatively, since

[2] Disregarding changes in protein turnover the predicted level of accumulation of hGH in these cells should be related to the integral of the transient expression (defined by the interval between medium changes).

this human cell line approximates but does not fully recapitulate the differentiation program of normal human enterocytes, it may produce "inappropriately" high levels of negative-acting transcription factors that serve to repress I-FABP$^{-1176\ to\ +28}$/hGH^{+9} and *Fabpi* expression.

A Nuclear Factor or Factors from Caco-2, but Not CV-1 Cells, Bind(s) to a 14-bp Repeated Element Present in Fabpi—The remarkable lineage specificity of I-FABP$^{-103\ to\ +28}$ expression observed *in vivo* led us to ask whether it binds gut-specific factors in nuclear extracts of small intestinal or colonic epithelial cells. When such extracts were prepared using a modification of the procedure of Gorski *et al.* (1986), we were able to identify a 24-bp element spanning nucleotides −212 to −188 that binds factors present in colonic but not small intestinal cells (Cohn *et al.*, 1992). Our functional mapping studies in transgenic mice indicated that this sequence is contained in a region of rat *Fabpi* that suppresses expression in ileum and colon (see Fig. 1 and Cohn *et al.* (1992)). When nuclear extracts were prepared from spleen, liver, kidney, proximal small intestine, and colon and used for electrophoretic mobility gel shift assays with labeled nucleotides −103 to +28, we were not able to identify any reproducible gut-specific binding patterns, despite the fact that control studies with the 24-bp sequence confirmed our earlier observations (data not shown).

Because nucleotides −103 to +28 of rat *Fabpi* maintain a striking degree of lineage specificity in the epithelial cell culture experiments and because of the difficulties in preparing active nuclear extracts from the gut due to contaminating luminal (pancreatic) proteases, we reasoned that nuclear extracts derived from cell lines that support or prohibit I-FABP$^{-103\ to\ +28}$/hGH^{+9} expression could be used to identify functionally important transcription factors that interact with its promoter elements. Therefore, extracts were prepared from Caco-2 cells and CV-1 cells immediately after they achieved confluency. Caco-2 extracts produced several retarded complexes with a labeled DNA fragment representing nucleotides −103 to +28 of rat *Fabpi* while no complexes were observed with nuclear extracts prepared from CV-1 cells (Fig. 3*A*, compare *lanes* 2 and 6). Binding of Caco-2 nuclear factors to I-FABP$^{-103\ to\ +28}$ was competed by a 50-fold molar excess of an unlabeled double-stranded oligodeoxynucleotide spanning bases −103 to −50 (Fig. 3*A*, *lane 3*) but not by a 50-fold molar excess of an oligodeoxynucleotide representing bases −49 to +1 (*lane 4*).

Nucleotides −103 to −50 of rat *Fabpi* contain a 14-bp element between bases −82 and −69 that is present in a comparable position in mouse and human *Fabpi* (Sweetser *et al.*, 1987; Green *et al.*, 1992). Each of these orthologous genes contains 2–3 additional copies of this element (consensus, 5′-TGAACTT(T/C)GAACTT-3′) located in similar positions of their 5′ nontranscribed domains (Green *et al.*, 1992; Cohn *et al.*, 1992; *cf.* Fig. 1). This 14-bp sequence is also found in the promoter regions of the homologous rat cellular retinol binding protein II gene and in several nonhomologous genes that are expressed in differentiating members of the enterocytic lineage (Demmer *et al.*, 1987; Sweetser *et al.*, 1987). An unlabeled oligodeoxynucleotide representing bases −82 to −69 of rat *Fabpi* was able to block binding of Caco-2 nuclear proteins to labeled I-FABP$^{-3\ to\ +28}$ DNA (Fig. 3*A*, *lane 5*).

By changing the buffer conditions used for the electrophoretic fractionation of these I-FABP$^{-103\ to\ +28}$ DNA-Caco-2 nuclear protein complexes, we were able to identify three distinct labeled bands. The formation of all three labeled complexes was blocked by a 50-fold molar excess of an oligodeoxynucleotide representing bases −103 to −50 or −82 to −69 of rat

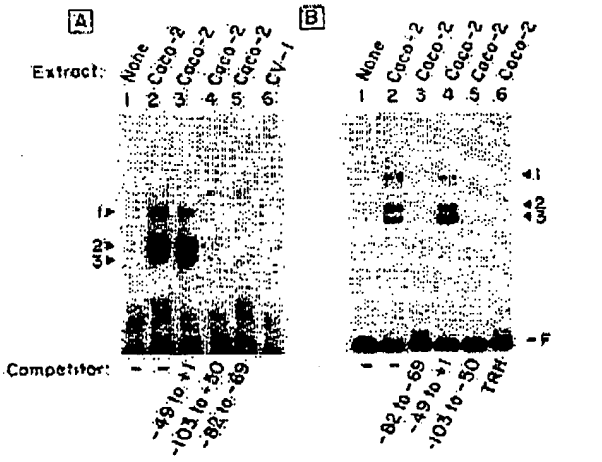

FIG. 3. **Formation of complexes between nuclear proteins present in Caco-2 cells and nucleotides −103 to +28 of rat *Fabpi*.** Nuclear extracts were incubated with 25 fmol of a ^{32}P-labeled DNA fragment spanning nucleotides −103 to +28 of rat *Fabpi* in the presence or absence of 1.25 pmol of the indicated oligodeoxynucleotide competitor. TRH refers to the HNF-4 binding site (5′-GCAAGGCTGAAGTCCAAGTTGAGTCC-3′) present in the promotor of the rat HNF-1 gene (Tian and Schibler, 1991). The reaction mixture was subjected to electrophoresis through 5% polyacrylamide gels using one of two buffer systems: 25 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.5 (*panel A*) or 45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0 (*panel B*). An autoradiograph of each gel is shown. The *arrows* point to three distinct DNA-protein complexes that form when nucleotides −103 to +28 of rat *Fabpi* are incubated with Caco-2 nuclear proteins. *F*, free I-FABP$^{-103\ to\ +28}$.

Fabpi but not by an oligodeoxynucleotide spanning bases −49 to +1 (Fig. 3*B*, compare *lanes 1–5*).

The Conserved 14-bp Element Spanning Nucleotides −82 to −69 Binds Hepatic Nuclear Factor-4 and Apolipoprotein Regulatory Protein-1—Previous searches of a data base of known transcription factor binding sites (Ghosh, 1990) with the 14-bp sequence had not revealed any significant matches. However, recent reports (Tian and Schibler, 1991; Kuo *et al.*, 1992) noted that the 5′ nontranscribed domain of the mouse and rat hepatic nuclear factor-1 (HNF-1) gene contains a sequence that binds hepatic nuclear factor-4 (HNF-4), a member of the steroid hormone receptor superfamily (Sladek *et al.*, 1990). This site in the HNF-1 gene (5′-GACTGAACTTTGGACTT-3′) contains 4 of 12 bases that differ from a previously reported consensus sequence for HNF-4 binding (5′-GGCAAAGGTCAT-3′; Sladek *et al.* (1990)). However, the HNF-1 gene's HNF-4 binding site (termed TRH) has only one mismatch with nucleotides −82 to −69 of rat *Fabpi* (Fig. 4). Moreover, an oligodeoxynucleotide representing this HNF-4 binding site is able to block formation of complexes between I-FABP$^{-103\ to\ +28}$ DNA and protein(s) present in Caco-2 nuclear extracts (Fig. 3*B*, *lane 6*).

Three observations led us to investigate whether HNF-4 or a related transcription factor could bind to the 14-bp sequence present between nucleotides −82 and −69 of rat *Fabpi* and what the functional consequences of such binding might be on expression of I-FABP$^{-103\ to\ +28}$/hGH^{+9} in Caco-2 and CV-1 cells. *First*, Mietus-Snyder *et al.* (1992) demonstrated that the human apolipoprotein (apo) CIII gene contains a sequence spanning nucleotides −86 to −74 that functions as a binding site for HNF-4 and two other closely related members of the steroid-thyroid hormone receptor superfamily of zinc finger transcription factors: apolipoprotein regulatory protein-1

ELEMENT		SEQUENCE
HNF-4 consensus		ATGACCTTTGCC
14 bp consensus		TGAACTTTGAACTT
ApoCIII C3P	−69	AGGTGACCTTTGCCCAGCG
HNF-1 TRH	−48	GACTGAACTTTGGACTT
rat *Fabpi* 1	−69	TCTTGAACTTTGAACTT
2	−432	TGTGCACTTTGAACCT
3	−579	CTTAAACTTT-AACTT
mouse *Fabpi* 1	−91	CCTGAACTTCGAACTT
2	−451	TGTGCACTTTGAAGTT
3	−571	TTTAACTTTGAAGTT
human *Fabpi* 1	−90	TCCTGAACTTTAAGCTT
2	−303	TCTGCACTTTGAACTT
3	−446	TATTAACTTT-AGCTT
4	−609	TCTTGAATTTTTGCTC
rat *Crbp1*	−502	TGAACTGTGACCTG
	−516	TGACCTGTGACCTG

FIG. 4. **Alignment of known HNF-4 binding sites in the HNF-1 and apoCIII genes with a conserved 14-bp sequence repeated in the proximal promoter regions of rat, mouse, and human *Fabpi* as well as rat *Crbp2*.** Alignments were generated according to Devereux *et al.* (1984). See text for discussion.

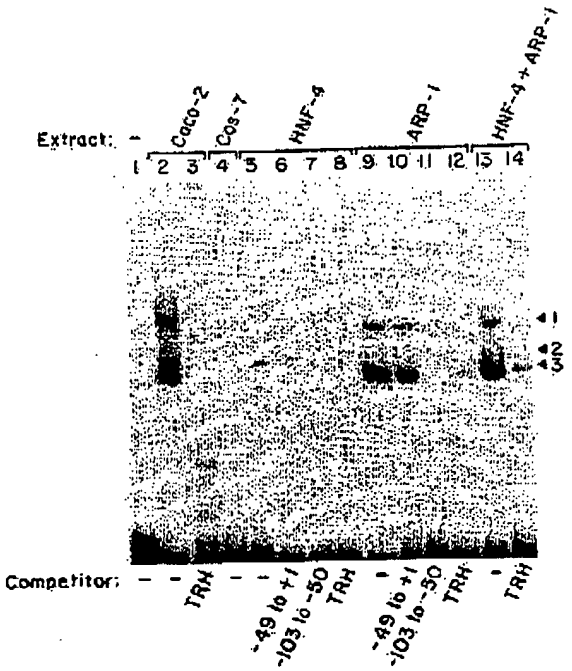

FIG. 5. **Evidence that the 14-bp sequence spanning nucleotides −82 to −69 of rat *Fabpi* binds HNF-4 and ARP-1.** Expression vectors encoding HNF-4 and ARP-1 (pMT2-HNF-4 and pMT2-ARP-1, respectively) were introduced into COS-7 cells. Cell lysates (1 μg of total protein), prepared 48 h after transfection, were incubated with 25 fmol of a ^{32}P-labeled DNA fragment encompassing nucleotides −103 to +28 of rat *Fabpi* in the presence or absence of 1.25 pmol of the indicated oligodeoxynucleotide competitor. The reaction mixtures were subjected to electrophoresis using an 8% polyacrylamide gel and the buffer conditions described in the legend to Fig. 3*B*. The *arrows* point to the three DNA-protein complexes that form with Caco-2 nuclear extracts. None are observed with a cell lysate obtained from COS-7 cells containing the pMT2 expression vector without insert. Bands 1 and 3 are observed with a lysate prepared from pMT2-ARP-1-transfected COS-7 cells and band 2 with pMT2-HNF-4-transfected COS-7 cell lysates. When ARP-1- and HNF-4-containing lysates are mixed (1 μg of total protein from each lysate), the pattern seen with Caco-2 nuclear extracts is largely recapitulated. (*N.B.* the concentration of ARP-1 and HNF-4 in lysates prepared from transfected COS-7 cells was not known.) *TRH*, HNF-1 gene's HNF-4 binding site.

(ARP-1, Ladias and Karathanasis (1991)) and Ear3/COUP-TF (Miyajima *et al.*, 1988; Wang *et al.*, 1989). Alignment of the ARP-1/HNF-4 binding site in the apoCIII gene and the consensus sequence of the 14-bp elements in rat *Fabpi* revealed that 10 of 14 bases are identical (Fig. 4). *Second*, studies conducted in HepG2 and Caco-2 cells showed that HNF-4 and ARP-1 bind to this cis-acting element in the human apoCIII gene (known as C3P) with similar affinities (Mietus-Snyder *et al.*, 1992). Co-transfection studies indicated HNF-4 and ARP-1 have opposing effects on apoCIII expression; HNF-4 functions as a C3P-dependent transcriptional activator in Caco-2 and HepG2 cells while ARP-1 binding represses transcription (Mietus-Snyder *et al.*, 1992). HepG2 and Caco-2 cells contain more HNF-4 than ARP-1 while CV-1 cells lack HNF-4 and ARP-1 (Mietus-Snyder *et al.*, 1992). *Finally*, *in situ* hybridization studies using late gestation mouse embryos revealed that HNF-4 and ARP-1 are co-expressed in hepatocytes and in epithelial cells located in intestinal crypts and villi (Mietus-Snyder *et al.*, 1992).

With these observations in mind, lysates were prepared from COS-7 cells transfected with a plasmid that directs expression of HNF-4 or ARP-1. Lysates prepared from cells transfected with the vector without insert DNA produce no retarded complex when incubated with nucleotides −103 to +28 of rat *Fabpi* (Fig. 5, *lane 4*). Lysates prepared from COS-7 cells transfected with the HNF-4 expression vector produce a single retarded complex that co-migrates with one of the three complexes produced by Caco-2 nuclear extracts (Fig. 5, compare *lanes 5* and *2*). Lysates prepared from COS-7 cells transfected with the plasmid encoding ARP-1 produce two distinct retarded complexes that co-migrate with the other two bands generated with Caco-2 nuclear extracts (Fig. 5, *lane 9*). Nucleotides −103 to −50 of rat *Fabpi* and an oligodeoxynucleotide derived from the TRH site in the HNF-1 gene block formation of the HNF-4·I-FABP$^{-103\ to\ +28}$ DNA complex (Fig. 5, *lanes 7* and *8*) and the ARP-1·I-FABP$^{-103\ to\ +28}$ DNA complexes (*lanes 11* and *12*). A 50-fold molar excess of an oligodeoxynucleotide derived from bases −49 to +1 of rat *Fabpi* has no effect on formation of complexes 1, 2, or 3 (*lanes 6* and *10* of Fig. 5).

When cell lysates prepared from COS-7 cells transfected with the HNF-4 or ARP-1 plasmids are mixed together and incubated with nucleotides −103 to +28 of rat *Fabpi*, three complexes are formed that have electrophoretic properties which mimic those produced with Caco-2 cell extracts (Fig. 5, *lanes 13* and *14*). These co-migrating complexes suggest, but do not prove, that HNF-4 and ARP-1 contained in Caco-2 extracts bind to nucleotides −82 to −69 of rat *Fabpi*. By using a methylation interference assay, we were able to demonstrate that the factor(s) responsible for forming the Caco-2·DNA complexes produce a pattern of protection of I-FABP$^{-82\ to\ -69}$, which is identical to that produced by the HNF-4- and ARP-1-containing COS-7 cell lysates (Fig. 6).

Co-transfection of Caco-2 and CV-1 Cells with I-FABP/hGH^{+1} DNAs and Plasmids Encoding either HNF-4 or ARP-

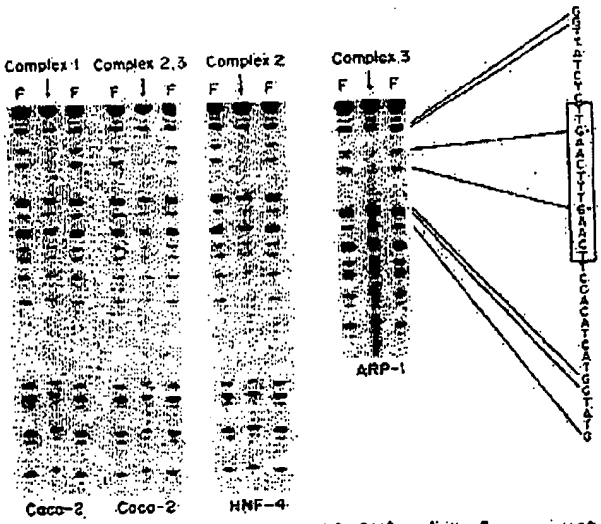

FIG. 6. **Comparison of the methylation interference patterns produced by Caco-2 nuclear extracts and COS-7 cell lysates containing HNF-4 and ARP-1.** A partially methylated DNA fragment spanning nucleotides −103 to +28 of rat *Fabpi* was incubated with nuclear extracts prepared from proliferating Caco-2 cells or cell lysates prepared from pMT2-ARP-1- or pMT2-HNF-4-transfected COS-7 cells. The upper band (*3*) and the two lower bands (*1* and *2*) produced with Caco-2 nuclear proteins were excised from a polyacrylamide gel similar to that shown in Fig. 3*B*. (The lower two bands were excised together since they could not be fully separated with sufficient reliability.) The more intense lower band, produced when pMT2-ARP-1-transfected COS-7 cell lysate was incubated with this DNA fragment (complex 3; see *lane 9* of Fig. 5), was excised from a gel as was the single retarded complex observed when pMT-HNF-4-transfected COS-7 cell lysate was incubated with nucleotides −103 to +28 (complex 2; see *lane 5* of Fig. 5). The radiolabeled I-FABP$^{-103\ to\ +28}$ fragment that was free of nuclear proteins was also excised (labeled *F* in Fig. 3*B*). The recovered material was subjected to guanosine-specific base cleavage using the method of Maxam and Gilbert (1977) and the products fractionated on a 15% polyacrylamide gel containing 8 M urea. The results indicate that the same two guanosines are protected in each complex. These guanosines occupy positions −74 and −81 within the conserved 14-bp element spanning nucleotides −82 to −69 of rat *Fabpi*.

1—The functional consequences of HNF-4 or ARP-1 binding to the 14-bp sequence were investigated. Because transcriptional effects can depend upon promoter context and transcriptional environment, several I-FABP/hGH^{+3} DNAs were introduced into Caco-2 and CV-1 cells together with the plasmids specifying HNF-4 or ARP-1. I-FABP$^{-277\ to\ +28}$/hGH^{+3}, I-FABP$^{-104\ to\ +28}$/hGH^{+3} and I-FABP$^{-103\ to\ +28}$/hGH^{+3} were used plus an additional construct containing two copies of the 14-bp element placed immediately upstream of nucleotide −277 of rat *Fabpi* (yielding I-FABP$^{E2/-277\ to\ +28}$/hGH^{+3}). This construct was designed to "amplify" any transcriptional effects specific to the 14-bp element and, in some sense, recreated the three repeats of this element found in I-FABP$^{-1178\ to\ +28}$/hGH^{+3} (Fig. 1). A parallel set of experiments was performed using a recombinant DNA that contained the C3P element of the human apoCIII gene fused to the thymidine kinase promoter and an hGH reporter (C3P-TK-hGH^{+3}).

Introduction of a HNF-4 expression vector into proliferating Caco-2 cells produced no significant change in the levels of hGH production by I-FABP$^{-103\ to\ +28}$/hGH^{+3}, I-FABP$^{-277\ to\ +28}$/hGH^{+3}, or I-FABP$^{E2/-277\ to\ +28}$/hGH+3 (Fig. 7*A*). Introduction of an ARP-1 expression vector produced no significant increase in expression of I-FABP$^{-103\ to\ +28}$/hGH^{+3},

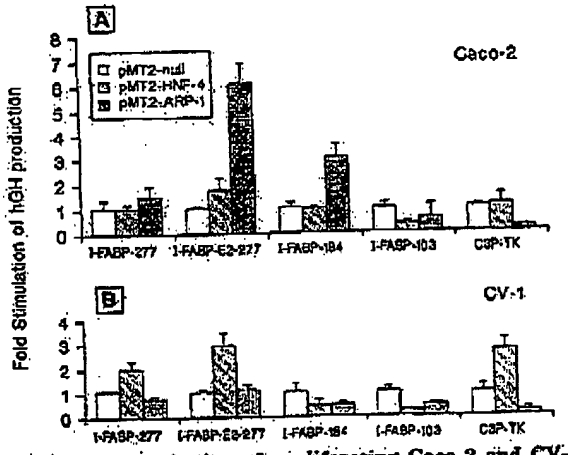

FIG. 7. **Co-transfection of proliferating Caco-2 and CV-1 cells with I-FABP/hGH^{+3} DNAs and plasmids encoding either HNF-4 or ARP-1.** hGH levels were determined in culture medium 64 h after transfection and expressed relative to the amount of β-galactosidase activity present in cell lysates prepared at the time medium was harvested. (RSV/β-Gal DNA was used as an internal standard to control for differences in transfection efficiency between experiments.) All experiments were repeated 4 times and the mean ± 1 S.D. plotted. Results are normalized to those obtained after co-transfecting cells with I-FABP/hGH^{+3} plus pMT2-null (the expression vector used to direct synthesis of ARP-1 or HNF-4).

a 3-fold increase in I-FABP$^{-184\ to\ +28}$/hGH^{+3} expression ($p < 0.005$ when compared with cells containing I-FABP$^{-184\ to\ +28}$/hGH^{+3} alone), a 1.5-fold increase in relative expression of I-FABP$^{-277\ to\ +28}$/hGH^{+3} ($p > 0.1$), and a 6-fold *increase* ($p < 0.005$) in relative expression of I-FABP$^{E2/-277\ to\ +28}$/hGH^{+3} (Fig. 7*A*).

Co-transfection of proliferating Caco-2 cells with pMT2-HNF-4 and C3P-TK-hGH^{+3} produced no significant change in the level of hGH production compared with the level observed in cells containing C3P-TK-hGH^{+3} alone. In contrast, co-transfection with pMT2-ARP-1 and C3P-TK-hGH^{+3} resulted in a 6–7-fold *reduction* in expression (Fig. 7*A*). Finally, co-transfection of proliferating Caco-2 cells with pMT2-ARP1 (or pMT2-HNF-4) and MT-hGH^{+3} produced no changes in hGH production (data not shown).

Together, these results support the notion that pMT2-ARP-1 changes the level of ARP-1 in proliferating Caco-2 cells and confirms the known response of the C3P element to this transcription factor (*i.e.* repression). The data also indicate that the 14-bp element located between nucleotides −82 and −69 of rat *Fabpi* can serve as a positive element when it binds ARP-1, although this latter effect appears to require and/or be influenced by sequence elements located between nucleotides −104 and −277 of rat *Fabpi*. While positive as well as negative transcriptional regulation by COUP-TF, a close relative of ARP-1, has been described (Wang *et al.*, 1987), prior co-transfection studies have only demonstrated a negative effect with ARP-1 (Mietus-Snyder *et al.*, 1992; Widom *et al.*, 1992; Cooney *et al.*, 1992; Tran *et al.*, 1992; Ladias *et al.*, 1992; Ladias and Karathanasis, 1991).

Unlike proliferating Caco-2 cells, pMT2-HNF-4 produces modest but significant increases in expression of I-FABP$^{-277\ to\ +28}$/hGH^{+3} and I-FABP$^{E2/-277\ to\ +28}$/hGH^{+3} in proliferating CV-1 cells, (levels of hGH production rise by 2–3-fold, $p < 0.005$; Fig. 7*B*). Removal of nucleotides −277 to −185 abolishes this stimulatory effect (Fig. 7*B*) while removal of nucleotides −277 to −104 converts the effect to a negative

one (hGH production falls 2–3-fold). The marked stimulatory effect of ARP-1 on I-FABP$^{E2/-277\ to\ +28}$/hGH^{+3} observed in proliferating Caco-2 cells was not observed in CV-1 cells where no change in hGH production was detectable (Fig. 7*B*). Additional control experiments demonstrated that co-transfection of proliferating CV-1 cells with pMT2-ARP1 and C3P-TK-hGH^{+3} produced a degree of relative suppression of hGH production similar to that observed in proliferating Caco-2 cells (Fig. 7, *A* and *B*). Finally, co-transfection with pMT-HNF-4 and C3P-TK-hGH^{+3} resulted in a significant (2-fold) stimulation of hGH production in proliferating CV-1 cells whereas no significant change was noted in proliferating Caco-2 cells (compare *panels A* and *B* in Fig. 7).

These results suggest the following conclusions. *First*, there appears to be a sufficient pool of HNF-4 present in proliferating Caco-2 cells so that any increase in the level of this transcription factor produced by pMT-HNF-4 has little detectable stimulatory effect on either C3P-TK-hGH^{+3} or I-FABP/hGH$^{E2/-277\ to\ +28}$/hGH^{+3} expression. Such a pool of functionally available HNF-4 does not appear to be present in CV-1 cells. Alternatively, Caco-2 cells may produce other factors, not represented in CV-1 cells, that can compete with HNF-4 for binding to C3P and a site or sites in I-FABP$^{E2/-277\ to\ +28}$/hGH^{+3}. Competition for binding to identical or highly conserved transcriptional regulatory sequences has been noted among various members of the steroid receptor superfamily of transcription factors that contain conserved, zinc finger DNA binding domains (Mistus-Snyder *et al.*, 1992; Widom *et al.*, 1992; Cooney *et al.*, 1992; Ladias *et al.*, 1992). *Second*, the fact that ARP-1 stimulates I-FABP$^{E2/-277\ to\ +28}$/hGH^{+3}, I-FABP$^{-277\ to\ +28}$/hGH^{+3}, and I-FABP$^{-104\ to\ +28}$/hGH^{+3} in proliferating Caco-2 but not CV-1 cells may reflect the presence or absence of these other competing transcription factor(s) and/or the fact that an endogenous ligand or ligands for this orphan receptor are present in one cell type but not another. *Third*, ARP-1 and HNF-4 appear to function as positive factors when bound to the conserved 14-bp element spanning nucleotides −82 to −69 of rat *Fabpi*. However, expression of such effects appears to require the participation of additional cis-acting elements located between nucleotides −277 and −104 and/or other transcription factors. Synergistic or cooperative effects between nearby elements have been demonstrated in a number of promoters (*e.g.* Kuo *et al.* (1990), Widom *et al.* (1991), and Strahle *et al.* (1988)). It is likely that a similar mechanism may be involved with *Fabpi*, *i.e.* I-FABP$^{-104\ to\ +28}$/hGH^{+3} but not I-FABP$^{-103\ to\ +28}$/hGH^{+3} is up-regulated by ARP-1 in Caco-2 cells even though both constructs contain a single copy of the 14-bp sequence. Nonetheless, based on results obtained in transgenic mice, these postulated sequences located between nucleotides −104 and −277 are not sufficient by themselves to support *Fabpi* expression in enterocytes; analysis of 1–20-week-old mice belonging to 10 pedigrees containing nucleotides −277 to −104 linked to a neutral promoter/reporter (the hGH gene beginning at its nucleotide −84) failed to disclose any hGH production in any intestinal cell lineage distributed along the duodenal-to-colonic axis (or in any one of nine extraintestinal tissues, Cohn *et al.* (1992)).

Perspectus—Functional mapping studies of the homologous rat *Fabpi* and liver fatty acid binding protein (*Fabpl*) genes in transgenic mice have indicated that distinct cis-acting elements regulate their patterns of expression along the crypt-to-villus and duodenal-to-colonic axes (Cohn *et al.*, 1992, Simon *et al.*, 1993). Moreover, these mapping studies have revealed that remarkably compact sequences located close to the start site of transcription (nucleotides −103 to −1 in *Fabpi* and nucleotides 132 to −1 in *Fabpl*) are apparently sufficient to establish and maintain appropriate cephalocaudal gradients of expression in enterocytes. However, comparative analysis of these sequences have failed to reveal any conserved domains other than their TATA boxes.

Further functional studies of these proximal promoter domains conducted in Caco-2 cells will have to be cautiously interpreted. Our comparison of the relative activities of I-FABP$^{-1173\ to\ +28}$/hGH^{+3}, I-FABP$^{-184\ to\ +28}$/hGH^{+3}, and I-FABP$^{-103\ to\ +28}$/hGH^{+3} in transgenic mice and Caco-2 cells emphasizes the differences in the transcriptional regulatory environments present in proliferating preconfluent and differentiated postconfluent Caco-2 cells and those present in members of the enterocytic lineage during their migration-associated differentiation program.

Formation of duodenal-to-colonic or crypt-to-villus gradients of *Fabpi* expression likely reflects a complex system that includes competition of multiple factors such as HNF-4 and ARP-1 for common cis-acting sequences.[3] Other transcriptional factors might also share this target sequence specificity. The conserved 14-bp element is quite similar to a direct repeat of the sequence TGACCT that serves as half of a "canonical" COUP-TF element (Kadowaki *et al.*, 1992). COUP-TF can compete with retinoic acid, thyroid hormone, and vitamin D_3 receptors for DNA binding and inhibit transcriptional activation (Cooney *et al.*, 1992; Tran *et al.*, 1992; Kliewer *et al.*, 1992; Widom *et al.*, 1992). Some of these other members of the transcription factor superfamily may also interact with the 14-bp site. Lineage-specific, differentiation-dependent, and regional differences in the relative abundance of these transcription factors could yield a rich repertoire of patterns of expression mediated by such proteins. Heterodimer formation, which is common among members of this transcription factor family (Forman and Samuels, 1990), would add exponentially to the flexibility of such a system. Furthermore the distribution of transcriptional co-factors (such as S300-II for COUP-TF, Tsai *et al.* (1987)) or endogenous ligands for these "orphan" receptors could affect their transcriptional properties. In addition, epigenetic modifications may contribute to *Fabpi*'s pattern of expression in the gut. We have found a gradient in the degree of methylation of *Fabpi* that parallels the level of its expression along the cephalocaudal axis.[4] Such epigenetic changes could represent an economical way to establish and maintain position information, *e.g.* by modifying the affinity of cis-acting elements for all of their cognate factors or for some specific subset of them. These considerations suggest that a combination of *in vitro*, cell culture, and *in vivo* experimental systems will be required to characterize exactly how the activity of this remarkably compact *Fabpi* promoter is regulated.

Acknowledgments—We thank our colleagues Burton Wice, Ted Simon, and Steve Cohn for the many helpful suggestions during this work and John A. Ladias (Departments of Medicine and Cellular and Molecular Physiology, New England Deaconess Hospital, Harvard Medical School, Boston, MA) for supplying expression plasmids for HNF-4 and ARP-1.

[3] A *Drosophila* homolog of HNF-4 has been described recently that is strikingly similar in structure and distribution to the mammalian protein (Zhong *et al.*, 1993). Deletion of the chromosomal region containing this gene prevents midgut development (Zhong *et al.*, 1993). These data underscore the central role that HNF-4 and related factors likely play in gut development and gene expression.

[4] J. N. Rottman and J. I. Gordon, unpublished observations. These differences in methylation are based on results obtained after *Msp*I and *Hpa*II digestion of DNA prepared from intestinal mucosa harvested at different positions along the duodenal-to-colonic axis.

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